

COMPARATIVE STUDIES ON MITOCHONDRIA ISOLATED FROM NEURON- ENRICHED AND GLIA-ENRICHED FRACTIONS OF RABBIT AND BEEF BRAIN

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ABSTRACT

Fractions enriched in neuronal and glial cells were obtained from dispersions of whole beef brain and rabbit cerebral cortex by large-scale density gradient centrifugation procedures. The fractions were characterized by appropriate microscopic observation. Mitochondria were then isolated from these fractions by differential centrifugation of their homogenates. The two different types of mitochondria were characterized with respect to certain enzyme activities, respiratory rate, rate of protein synthesis, and their buoyant density in sucrose gradients. The mitochondria from the neuron-enriched fraction were distinguished by a higher rate of incorporation of amino acids into protein, higher cytochrome oxidase activity, and a higher buoyant density in sucrose density gradients. Mitochondria from the glia-enriched fraction showed relatively high monoamine oxidase and Na^+ - and K^+ -stimulated ATPase activities. The rates of oxidation of various substrates and the acceptor control ratios did not differ appreciably between the two types of mitochondria. The difference in the buoyant density of mitochondria isolated from the neuron-enriched and glia-enriched cell fractions was utilized in attempts to separate neuronal and glial mitochondria from the mixed mitochondria obtained from whole brain homogenates in shallow sucrose gradients. The appearance of two peaks of cytochrome oxidase, monoamine oxidase, and protein concentration in such gradients shows the potential feasibility of such an approach.

INTRODUCTION

The development of methods for separating whole brain dispersions into neuron-enriched and glia-enriched fractions makes possible more direct biochemical analysis of the properties and metabolic characteristics of these two types of cells, which much evidence suggests are functionally interrelated (Hyden, 1967). Of the various methods advanced for separation of neurons and glia (Korey, 1958; Satake and Abe, 1966; Bocci,

1966; Rose, 1965, 1967; Cremer et al. 1968; Blomstrand and Hamberger, 1969; Norton and Podusio, 1969), centrifugal separation methods employing a discontinuous sucrose-Ficoll density gradient appear to be most successful in minimizing contamination of the fractions by detached synaptic nerve endings and other structures (Blomstrand and Hamberger, 1969). However, since the neuronal and glial cell fractions still contain some con-

taminating material, they will be referred to as neuron-enriched and glia-enriched fractions, respectively. Application of these methods to large amounts of tissue has not been reported.

This study describes the isolation and characterization of mitochondria from neuron-enriched and glia-enriched fractions obtained from rabbit and beef brain by a discontinuous gradient method described earlier (Blomstrand and Hamberger 1969), modified to handle the much larger quantities of tissue required for the mitochondrial isolations. Data on the ATPase, cytochrome oxidase, and monoamine oxidase activities of the neuronal and glial mitochondria are given, as well as a comparison of respiratory activity, acceptor control ratios, and capacity for protein synthesis. It is also shown that these two types of mitochondria differ significantly in their rate of sedimentation; peaks corresponding to mitochondria from neuronal and glial fractions can be distinguished in mixtures of whole brain mitochondria subjected to sucrose density gradient centrifugation.

MATERIALS AND METHODS

Recovery of Neuron- and Glia-Enriched Fractions from Whole Cerebral Cortex

RABBIT BRAIN: Albino rabbits weighing 1.5–1.8 kg were killed by infusion of Ringer's solution via a cannula into the left ventricle of the heart inserted under Nembutal anaesthesia. The cerebral cortex was chopped twice in planes perpendicular to each other in a McIlwain tissue chopper (Mickle Laboratory Engineering Co., England) set at 0.4 mm. The tissue was disrupted in 3 ml of suspending medium per cortex by passing the suspension up and down 15 times through a 1 mm nylon mesh cemented to the end of a 10 ml syringe from which the needle support had been removed. After this procedure, the disrupted brain tissue was suspended in 40–50 ml of the medium (0.32 M sucrose, 10 mM Tris-HCl buffer pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 2% Ficoll [w/v] [Pharmacia Fine Chemicals Inc, New Market, N.J.]).

The suspension was then successively sieved through nylon mesh having openings of 1000, 120, 75, and 50 μ ; the last mesh was used in a double layer. The sieved suspension was centrifuged at 150 g for 5 min. The supernatant fraction was discarded, and the bottom layer was used for further fractionation.

A discontinuous gradient was prepared in the following manner. First, 5.0 ml 40% sucrose (w/w) was added to the tube, and on this were placed

successive layers of 5.0 ml 30% Ficoll, 15.0 ml brain suspension in 20% Ficoll, 5.0 ml 15% Ficoll, and finally 5.0 ml 10% Ficoll. The tubes were spun immediately at 54,000 g for 120 min in an SW 25 rotor in a Beckman Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting bands at the top, the bottom, and the interfaces were collected with a Pasteur pipette, resuspended in a large volume of 0.32 M sucrose, and collected by centrifugation at 20,000 g for 10 min. The neuron-enriched fraction collects at the interface between 30% Ficoll and 40% sucrose, whereas the glia-enriched fraction collects at the interface between 10 and 15% Ficoll. The layer on top of the 10% Ficoll layer consists largely of myelin and nerve fiber material, and to a certain extent of glial cells. The pellet is composed of free nuclei from different cell types and a small number of neuronal perikarya. The layers above and below the 20% Ficoll contain mixed cell populations and small blood capillaries.

BEEF BRAINS: Beef brains were obtained within 1 hr after slaughter of the animals and transported to the laboratory in plastic bags surrounded by crushed ice. The cerebral cortex was freed from as much white matter as possible, passed twice through a cold meat grinder at a medium setting, and suspended 10% (w/v) in a medium containing sucrose (0.32 M), Tris chloride pH 7.6 (10 mM), heparin (Liquaemin sodium 100; 10,000 USP units/l), and EDTA in a concentration of 0.5 mM. Ordinarily batches of from 500 to 1000 g of minced beef brain were carried through the entire procedure. The suspension was stirred at 1000 rpm with a blunt polyethylene propeller for 30 min at 4–7°C. The dispersed cells were filtered through nylon bolting cloth (Tobler, Ernst, and Trabler, New York) in three successive steps, with mesh openings of 1.0, 0.2, and 0.1 mm successively. The suspension was then passed through two stainless steel sieves having openings of 88 and 63 μ (U.S. Standard Sieve Series No. 170 and 230) in succession, to remove clumps of undispersed cells. The filtered suspension was then freed from other debris, including free mitochondria, by centrifuging the cells down at 350 g for 5 min in 250 ml polyethylene bottles. The supernatant fraction, which represented 90% of the total volume and which contained free mitochondria, was siphoned off and discarded. The remaining concentrated cell suspension at the bottom of the bottles was recovered, resuspended in a volume of fresh, cold medium equal to that employed in the first washing, and centrifuged again at 350 g for 5 min. After removal of the supernatant medium, the washed cells (200 ml, containing about 3.0 g protein) were mixed with 0.67 volume (\sim 133 ml) of fresh medium containing Ficoll (30% w/v). The final Ficoll concentration was thus 12%.

Aliquots (25–30 ml) of the sucrose-Ficoll suspension of brain cells (volume = 330 ml, containing around 3 g of protein) were layered on 6 ml of 1.2 M sucrose in cellulose nitrate tubes and centrifuged in the No. 30 rotor of the Spinco Model L centrifuge for 60 min at 60,000 *g*. The neuron-enriched fraction collected at the top of the 1.2 M sucrose layer and the glia-enriched fraction on top of the 12% Ficoll layer.

Preparation of Mitochondria

Whole cerebral cortex, or the glial and neuronal cell fractions obtained therefrom, were homogenized in 9 volumes of cold 0.32 M sucrose containing 0.01 M Tris-HCl (pH 7.4); standard glass-Teflon homogenizers (Arthur H. Thomas Co., Philadelphia, Pa.) were employed at a speed of 1400–1500 rpm. The homogenates were centrifuged twice at 900 *g*, the pellets discarded, and the resulting supernatant fractions centrifuged at 10,000 *g* to yield the mitochondrial fractions. The pellets were washed three times; the top layer was discarded each time. The mitochondria were finally collected as pellets by centrifugation at 6,500 *g* for 10 min.

Subfractionation of Crude Mitochondrial Fractions

For some experiments the mitochondria were further purified on a discontinuous sucrose gradient according to Gray and Whittaker (1962). The mitochondria were suspended in 0.32 M sucrose (20–30 mg protein/ml), layered on a discontinuous gradient of 0.8 M and 1.2 M sucrose, and centrifuged for 30 min in the SW 25 rotor (Spinco) at 54,000 *g*. The resulting pellet was recovered and resuspended in 0.32 M or 0.80 M sucrose.

Buoyant Density Comparison of Brain Mitochondria in Sucrose Density Gradients

Continuous sucrose gradients (20–50%, 30–50%, 35–48% w/w) were formed in tubes (Buchler Instruments, Inc., Fort Lee, N.J.) and kept cold for 2–3 hr before use. Aliquots (2.0 ml) of the mitochondrial suspension in 0.32 M sucrose (2–3 mg protein/ml) were applied to the top of the gradients and centrifugation carried out for 120 min at 54,000 *g*. In some experiments a 15 hr centrifugation time was used, with essentially no difference in relative position of the bands. The tubes were drained into a continuous flow cell, and the protein content was monitored at 280 μ . The concentration of the sucrose was checked in each 1.0 ml fraction by refractometry.

Enzymatic and Chemical Assays

Prior to assay of individual enzymes, all mitochondrial fractions were subjected to sonic irradiation for 1.0 min at 0°C in order to release maximal activity.

CYTOCHROME OXIDASE: Activity was assayed polarographically by measuring the oxygen consumption with a Clark electrode at 30°C in an assay system containing 75 mM phosphate buffer (pH 7.4), 30 μ M cytochrome *c* (horse heart Type III, Sigma Biochemical Co., St. Louis, Mo.), 3.75 mM sodium ascorbate, and 0.3 mM *N, N, N', N'*-tetramethyl-*p*-phenylenediamine hydrochloride (TMPD).

MONOAMINE OXIDASE: Activity was assayed radiometrically by the method of Wurtman and Axelrod (1963) using tyramine-¹⁴C as substrate or spectrophotometrically by a modification of the method of Tabor et al. (1954), by following the formation of benzaldehyde at 250 μ M at 30°C in an assay system containing 2.5 mM benzylamine hydrochloride as substrate and 50 mM phosphate buffer, pH 7.6.

GLUTAMATE DEHYDROGENASE: Activity was determined by measuring the oxidation of NADH spectrophotometrically at 366 μ M, slightly modified after Schmidt (1963).

LACTIC DEHYDROGENASE: Activity was measured spectrophotometrically according to Wroblewski and La Due (1955).

ADENOSINE TRIPHOSPHATASE: Activity was assayed in a reaction mixture containing 30 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 5.0 mM Tris-ATP. When added, NaCl was 120 mM and KCl 20 mM. The 1.0 ml system contained 0.2–0.4 mg protein. The incubation time was 10 min at 37°C. The reaction was stopped by adding 0.1 ml 50% (w/v) trichloroacetic acid (TCA), and the tubes were transferred to ice. Inorganic phosphate was determined colorimetrically (Gomori, 1942).

RESPIRATORY RATE AND ACCEPTOR CONTROL RATIOS: Respiratory rate and acceptor control ratios were assayed polarographically on intact mitochondria by measuring the oxygen consumption with a Clark electrode in an assay system containing 0.3 M mannitol, 10 mM KCl, 10 mM Tris-HCl buffer (pH 7.4), 5.0 mM K-phosphate buffer (pH 7.4), and 2 mg/ml bovine serum albumin. All substrates were added to a final concentration of 10 mM, except malate, which was added at 5.0 mM. ADP was added at 20–50 μ M to initiate the State 4—State 3 transition.

INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO MITOCHONDRIAL PROTEIN: These measurements were carried out on intact mitochondria in the system of Wheeldon and Lehninger (1966). The incubation was carried out for 60 min

at 37°C. The reactions were stopped by addition of 1.0 ml ice-cold 10% TCA to the 1.0 ml incubation system. The precipitated material was washed three times in 5% TCA and then suspended in 5% TCA and heated at 90°C for 20 min. The cooled precipitate was then extracted twice with ether-ethanol (4:1 v/v) to extract lipids. The final precipitate was dissolved in formic acid and counted in a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Protein was determined according to Murphy and Kies (1960) or Lowry et al. (1951).

MORPHOLOGICAL EXAMINATION OF THE FRACTIONS. All fractions were examined routinely by phase contrast and following staining with methylene blue. For more permanent preparations the fractions were pelleted, fixed in buffered 10% formalin, paraffin-embedded, and the sections were stained either with toluidine blue, the gold chloride sublimate method of Cajal (Romeis, 1948), or by Palmgren's

(1948) method for axons. The best results were obtained when the suspensions from the gradients were collected on Millipore filters after fixation in 50% ethanol. The filters were then stained with routine cytological methods such as the Papanicolaou stain (Hamberger, Norrby, and Ericsson, in preparation). Figs. 1 and 2 show light micrographs of the neuron-enriched and glia-enriched fractions.

For electron microscopy, samples of mitochondria that had been purified on discontinuous sucrose gradients (see above) were fixed by placing a small drop of the suspension on the inside surface of the centrifuge tube, above the surface of a small volume of ice-cold 6.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The contents were quickly mixed and centrifuged for 4 min at top speed in a Beckman Microfuge. All subsequent steps were carried out at 0–4°C. The pellets were removed from the Microfuge tubes and washed three times with 0.25 M sucrose in 0.1 M phosphate buffer, pH 7.2. They were fixed for

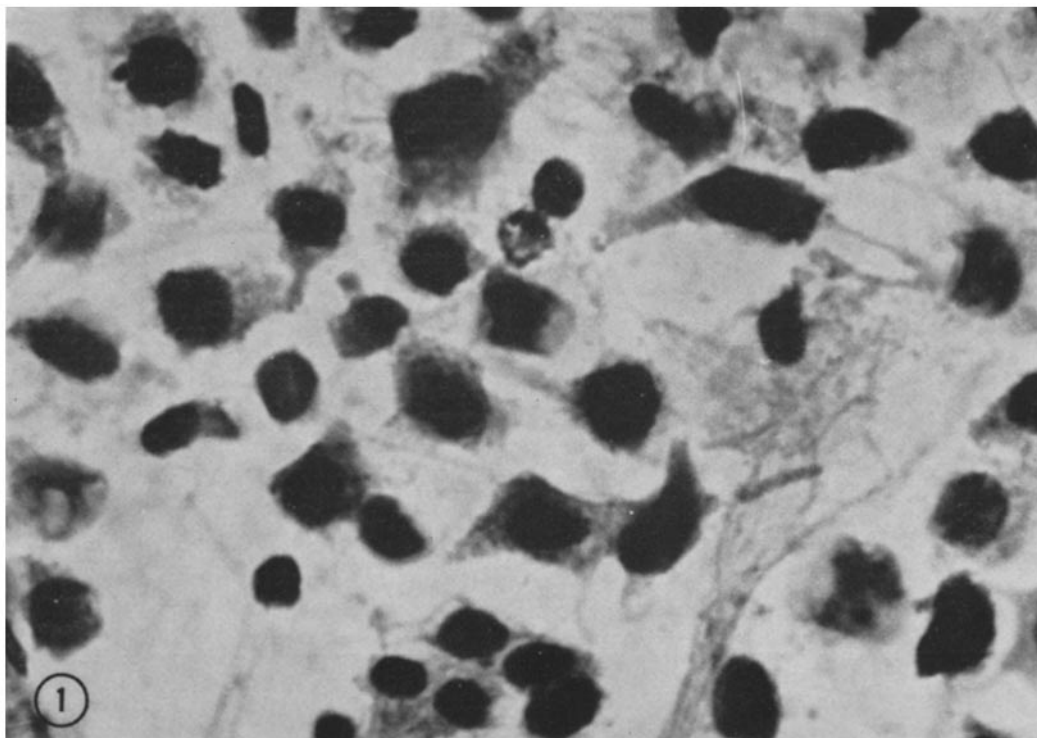


FIGURE 1 Photomicrograph of neuron-enriched fraction. An aliquot of the fraction was removed from the preparative gradient by means of a Pasteur pipette, diluted approximately 10-fold with 0.32 M sucrose containing 0.1 M NaCl, and fixed in 50% ethanol. The cells were collected from the suspension on a Millipore filter by gentle suction and stained according to Papanicolaou. The filter was then cleared with toluene, mounted, and photographed under the light microscope. Most of the structures present are neuronal perikarya with proximal dendrites. A few small and dark glial cell nuclei and some fibrous material are seen as contaminants of the fraction. $\times 1000$.

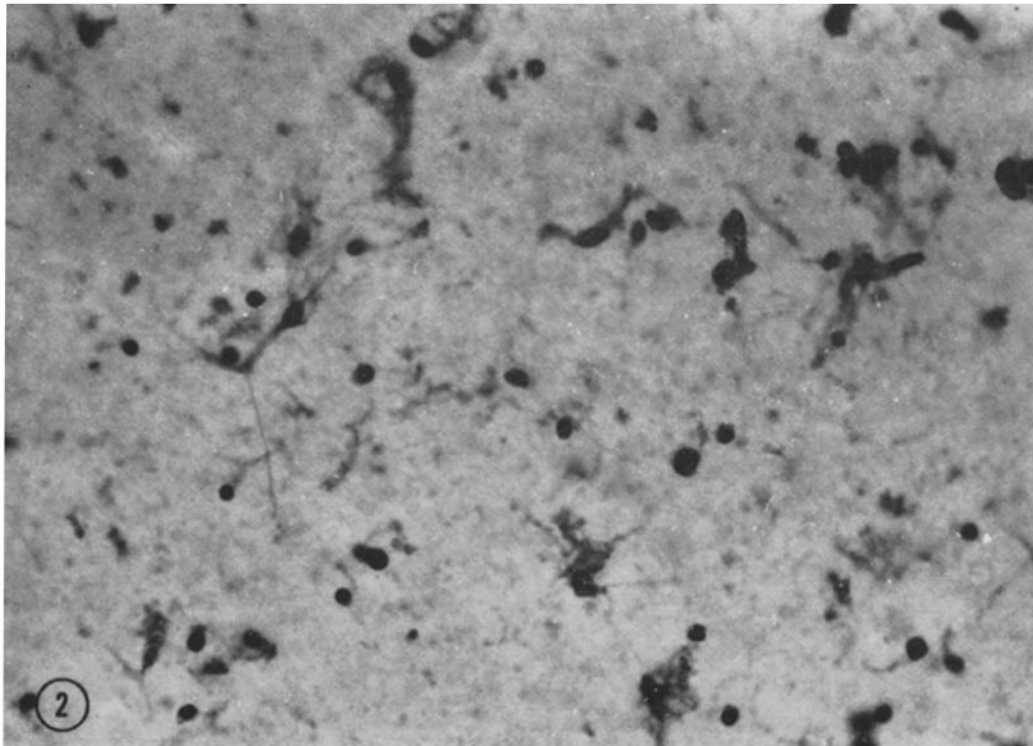


FIGURE 2 Photomicrograph of glia-enriched fraction. Technical details as in Fig. 1. 10-12 relatively intact astrocytes are seen (oval dark nuclei and long, slender cell processes). A large number of oligodendroglial cells (round, dark nuclei) are present, with a small rim of cytoplasm around the nucleus. $\times 400$.

1-1.5 hr in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.2. The fixed pellets were dehydrated by passage through an ethanol series and embedded in Epon 812 by the procedure of Luft (1961). The pellets were sectioned with a diamond knife on an LKB Ultramicrotome, (LKB Instruments Inc., Rockville, Md.) and the sections were collected on unsupported 400-mesh grids. They were examined in a Siemens Elmiskop I operated at 80 kv and photographed at plate magnifications of $\times 18,000$.

RESULTS

Preparation of Whole Cell Suspensions from Brain

In exploratory investigations it was found that procedures satisfactory for preparing suspensions of liver cells do not give correspondingly good results with brain tissue. Some attempts were made to obtain a better suspension medium by varying

its composition and by addition of citrate, sodium tetraphenylborate (Rappaport and Howze, 1966) proteolytic enzymes, and other solutes. However, none of these variations was clearly superior; in most experiments we used the simple sucrose media described in Materials and Methods. All procedures were carried out in polyethylene containers; glassware was avoided to minimize cell disruption due to adhesive forces.

During the disruption and sieving of brain tissue, large numbers of both neuronal and glial cells are broken, with release of free mitochondria of both kinds. Unless they are removed, they will appear as contaminants of the unbroken neuron- and glia-enriched cell fractions, and thus of the mitochondrial fractions isolated from them. Removal of such free mitochondria was accomplished by the low speed centrifugation step preceding the separation of neuron-enriched and glia-enriched fractions on the Ficoll-sucrose gradient.

Preparation of Neuron-Enriched and Glia-Enriched Fractions

The large-scale method employed to obtain cell-enriched fractions from beef brain differed in some steps from the method used for rabbit brain. One problem was the limited amount of material that can be separated on a discontinuous gradient with a SW 25 rotor (Spinco Model L). Large capacity zonal rotors (B. IV) were also employed, but the inability of the equipment available to us to maintain a sharply discontinuous step-gradient made it impossible to obtain good separations on a large scale. The large capacity needed for the beef brain separations was most simply met by the No. 30 rotor (Spinco). Although this is an angle-head rotor, it was found to be very effective. The final concentration of Ficoll that gave optimal separation of the cell fractions from beef brain was about 12%. There is evidently a species variation with respect to behavior of brain cells in such gradients, since it was necessary to use a considerably higher Ficoll concentration in order to obtain a similar separation of neuronal and glial cells from rabbit cerebral tissue.

Yield of Neuronal and Glial Fractions

The amount of starting material used was around 3–4 g for one rabbit brain and 800–1000 g for one beef brain (wet weight). From gradients loaded with material from one rabbit brain, the glia-enriched fraction (around 6 mg protein) was collected between 10 and 15% Ficoll and the neuron-enriched fraction (around 3 mg protein) between 30% Ficoll and 40% sucrose. In the somewhat simpler gradient used for beef brains, the neuron-enriched fraction (around 200 mg protein) was collected on top of the 1.2 M sucrose layer, and the glia-enriched fraction (around 2000 mg protein) on top of the 12% Ficoll layer.

The neuron-enriched cell fraction (Fig. 1) contained largely nerve cell bodies; the major contaminants were free neuronal nuclei and some small blood capillaries. The fraction enriched in glial cells (Fig. 2) contains oligodendrocytes and astrocytes demonstrable by the Cajal method. However, the fraction also contains some unidentified material, probably neuronal cell processes. The fractions obtained from rabbit brains had generally a somewhat better morphological appearance than the material from beef brain. The contaminants of such fractions have

been discussed in detail elsewhere (Blomstrand and Hamberger, 1969).

Yields and Appearance of Mitochondria

The final yield of mitochondrial protein, following isolation and purification of the mitochondria by density-gradient centrifugation, is about 10–20 mg for the case of neuronal mitochondria and somewhat higher for glial mitochondria per beef brain. A corresponding yield was obtained for the rabbit brain fractionation.

Electron microscopy revealed that both types of mitochondria were obtained in a relatively good state of preservation, with distinct membranes and somewhat varying configuration of the internal structure. Some outpocketing and breakage of the outer membranes was observed. There was no conspicuous difference in size, electron opacity, or conformation of the cristae in the mitochondria from the neuron-enriched and glia-enriched fractions (Figs. 3 and 4). Both showed approximately the same admixture of synaptic nerve endings. Very little contamination by other nonmitochondrial elements was found.

Results of Enzyme and Metabolic Studies

ATPASE ACTIVITY OF WHOLE CELL HOMOGENATES: Adenosine triphosphatase activity was determined in unfractionated homogenates of the neuron-enriched and glia-enriched fractions from beef brain. The activities were very similar in both fractions. The presence of Na^+ and K^+ stimulated both fractions by about 100% (Table I)

ATPASE ACTIVITIES OF MITOCHONDRIA: In the presence of Mg^{++} alone, no difference in the ATPase activity between the mitochondria from beef brain nerve and glial cell fractions was observed (Table II). However, the $\text{Na}^+ + \text{K}^+$ -stimulated activity was significantly higher in the mitochondria from the glial cell fraction.

CYTOCHROME OXIDASE AND MONOAMINE OXIDASE ACTIVITIES: Cytochrome oxidase and monoamine oxidase activities were about 20% higher in the mitochondria from the glial cell fraction of beef brain than in those from nerve cells (Table III). The neuronal mitochondria from rabbit brain had a slightly higher cytochrome oxidase activity, while monoamine oxidase activity of the glial mitochondria was much higher than in the neuronal mitochondria.

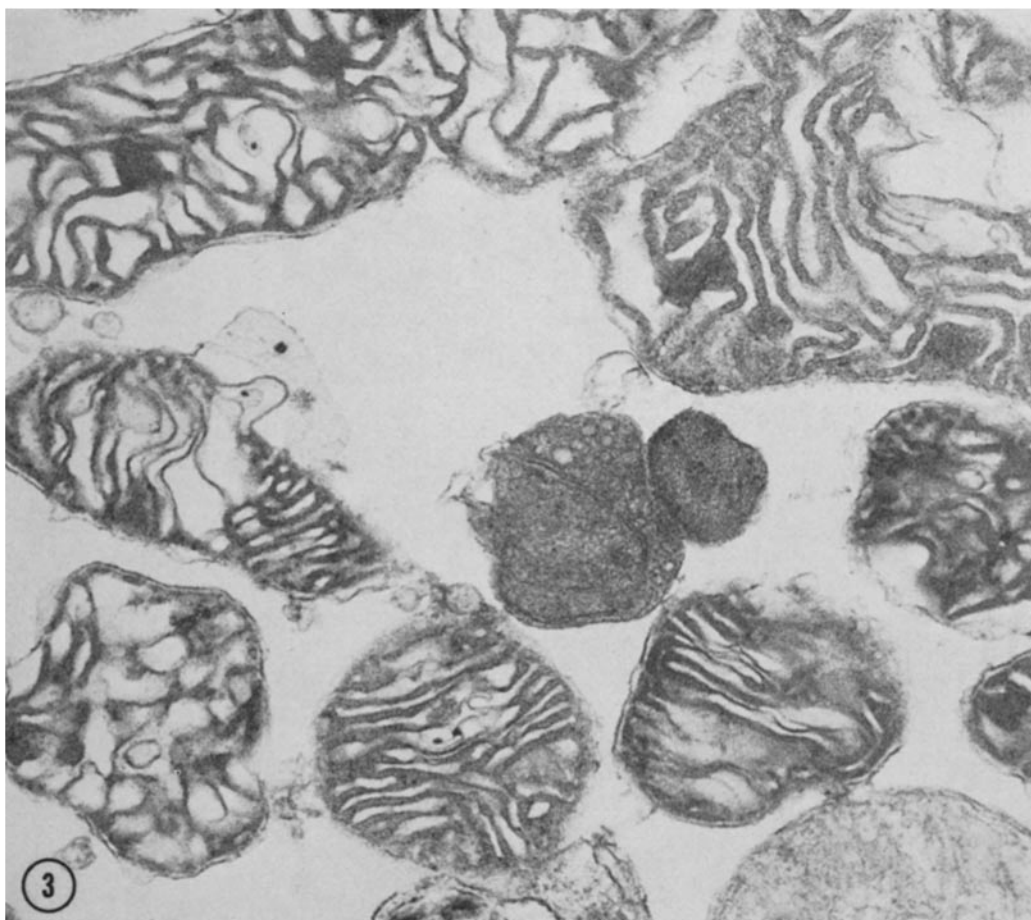


FIGURE 3 Electron micrograph of mitochondria from neuron-enriched fraction. $\times 56,000$.

RESPIRATORY RATE: The respiration studies were performed with bovine serum albumin (2 mg/ml) included in the isolation medium in order to insure maximal acceptor control ratios. Of the respiratory substrates tried, succinate supported the highest rate of State 3 oxygen consumption in both nerve cell and glial mitochondria, which showed approximately equal rates. In neuronal mitochondria, pyruvate + malate and α -glycerophosphate were oxidized at rates of 40 and 85%, respectively, of the rate with succinate; in glial cell mitochondria, pyruvate + malate was oxidized at 50% and α -glycerophosphate at 65% of the rate of succinate oxidation.

ACCEPTOR CONTROL RATIOS: The acceptor control ratio was 1.5–2.0 for succinate and 2.0–3.0 for pyruvate + malate in most preparations of

both types of mitochondria; no significant difference was observed between glial and neuronal mitochondria of beef brain.

Protein Synthesis

Mitochondria prepared from neuronal and glial cell fractions and purified on a discontinuous sucrose gradient were compared with respect to their capacity to incorporate L-leucine- ^{14}C into mitochondrial proteins in an *in vitro* system. Table IV shows the results for beef brain mitochondria. The conditions of incubation were varied somewhat in the different experiments (Wheeldon and Lehninger, 1966). In all experiments, the neuronal mitochondria incorporated leucine at nearly twice the rate shown by glial

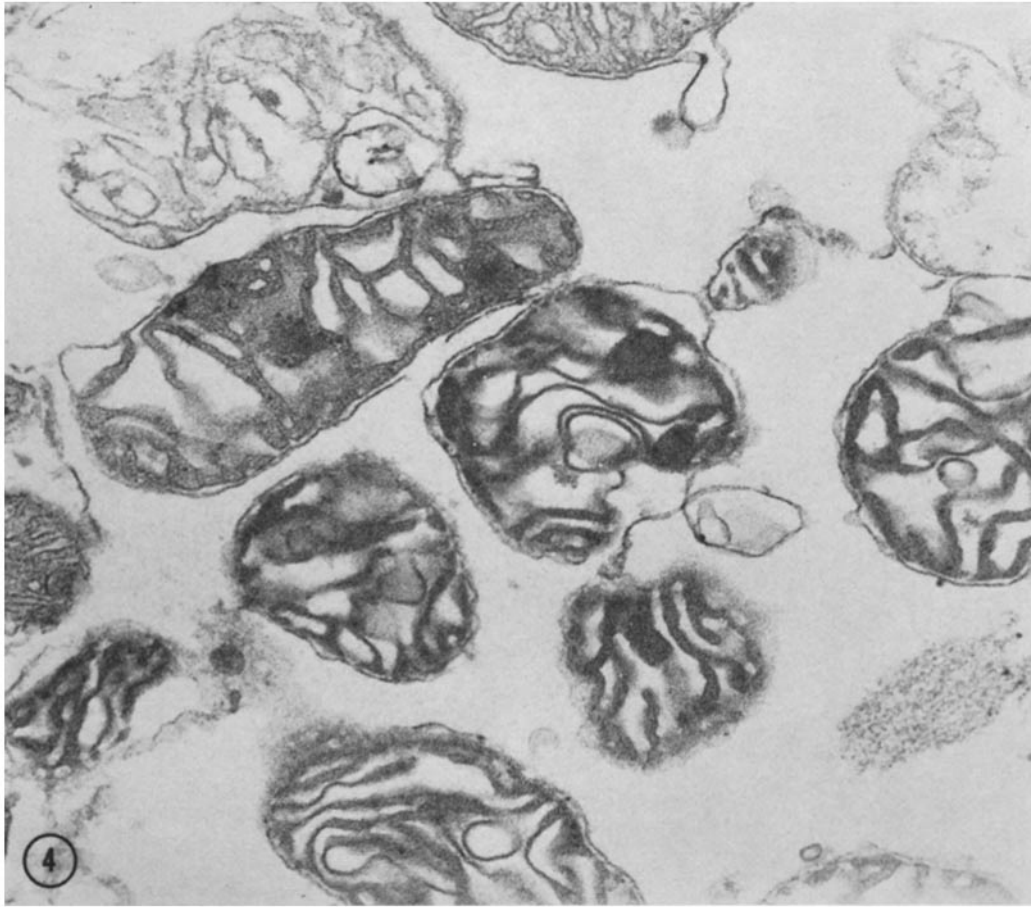


FIGURE 4 Electron micrograph of mitochondria from glia-enriched fraction. $\times 56,000$.

mitochondria. Incorporation of leucine was stimulated by an ATP generating system, and also by addition of ammonium sulfate, as previously shown in the case of heart mitochondria (Hamberger et al., 1969). Neuronal and glial mitochondria from beef and rabbit showed corresponding differences with respect to amino acid incorporation.

Density Gradient Patterns of Mitochondria from Neuron- and Glia-Enriched Fractions

Mitochondria previously purified on a discontinuous sucrose gradient according to Gray and Whittaker (1962) were centrifuged on a continuous sucrose gradient as described in Materials and Methods. The sedimentation pattern for beef brain mitochondria is shown in Fig. 5.

The mitochondria isolated from the neuronal fraction are present in the peak at 39.5% sucrose. In contrast, mitochondria isolated from the glial cell fraction showed a peak at 38.5% sucrose. Both these peaks fall within the somewhat broader peak of the mixed mitochondria isolated from whole unfractionated brain cells (Fig. 5). The density difference between the mitochondria of nerve cells and those from glial cells was consistently observed, even when the conditions and time of centrifugation were varied widely. The absolute position of the peaks was somewhat lower in the gradient after longer centrifugation periods, but their relative positions remained unchanged. The differences in buoyant density of the two types of mitochondria were not great enough to produce resolution of two peaks in the sucrose density gradient profile of mixed whole

TABLE I

Adenosine Triphosphatase Activities in Homogenates of Neuron- and Glia-Enriched Fractions of Beef Brain
The activity is expressed as μ moles phosphate formed/10 min per mg protein at 37°C. Numbers in parentheses in this and the following tables indicate the number of preparations analyzed. The standard error of the mean is given in each case.

	Without Na ⁺ and K ⁺	Additional activity in the presence of Na ⁺ and K ⁺
Neuronal fraction	0.69 \pm 0.03 (6)	0.65 \pm 0.04 (6)
Glial fraction	0.58 \pm 0.05 (6)	0.62 \pm 0.06 (6)

TABLE II

Adenosine Triphosphatase Activities of Mitochondria from Neuronal and Glial Cell Fractions of Beef Brain
The mitochondria were purified on a discontinuous sucrose gradient and pretreated with Lubrol WX prior to assay

	Without Na ⁺ and K ⁺	Additional activity in the presence of Na ⁺ and K ⁺
Neuronal fraction	0.86 \pm 0.10 (9)	0.24 \pm 0.07 (3)
Glial fraction	0.86 \pm 0.03 (9)	0.53 \pm 0.06 (3)*

*P < 0.005.

TABLE III

Cytochrome Oxidase and Monoamine Oxidase Activities in Mitochondria from Neuronal and Glial Fractions of Beef Brain

The mitochondria were purified on a discontinuous sucrose gradient and pretreated with Lubrol WX. Cytochrome oxidase activity is expressed in μ atoms O₂ consumed/min per mg protein. Monoamine oxidase is expressed as the change in log I₀/I at 250 nm at 30°C/5 min per mg of protein in a 2.1 ml system (see text).

	Cytochrome oxidase	Monoamine oxidase
Neuronal fraction	0.865 \pm 0.83 (12)	0.0485 \pm 0.007 (11)
Glial fraction	1.066 \pm 0.149 (12)	0.0595 \pm 0.005 (11)

Results are given with the standard error of the mean.

beef brain mitochondria centrifuged under the same conditions, as is also shown in Fig. 5. However, as is seen below, better separation was possible using rabbit brain preparations.

Centrifugation in sucrose density gradients of mitochondria isolated from neuronal and glial cell fractions of rabbit brain cortex again showed that the neuronal mitochondria have a greater buoyant density. The mitochondria from the glial cell fraction showed a peak at 38.5% sucrose, with a shoulder at 41%, whereas the neuronal mitochondria showed a broad peak with a maximum at about 43% sucrose (Fig. 6). However, the mitochondrial fraction from the rabbit brain glial cells is grossly contaminated with synaptic nerve endings. This is strongly suggested by the

position of the peak of mixed whole rabbit brain mitochondria at about 36% sucrose (Fig. 6), which evidently contains considerable light non-mitochondrial material. Contamination by synaptic endings is more directly demonstrated by the results of monoamine oxidase and cytochrome oxidase assays on the separated mitochondria from neuron-enriched and glia-enriched fractions of rabbit brain (Fig. 7). The cytochrome oxidase and monoamine oxidase activities yield congruent peaks at 42.5% sucrose for glial mitochondria and at 44% sucrose for neuronal mitochondria, confirming the results of Fig. 6. (The somewhat higher apparent density of both peaks in Fig. 7 is due to a longer centrifugation time). However, at the light end of the glial peak, cytochrome oxi-

TABLE IV
Incorporation of Leucine-¹⁴C into Protein of Mitochondria from Nerve and Glial Cell Fractions of Beef Brain

The isolation of mitochondria is described in the text. The incubation was carried out at 37°C for 60 min in a 1 ml system, with about 0.5 mg mitochondrial protein per incubation vessel. The medium contained Tris-HCl buffer, pH 7.4 (30 mM); KCl (50 mM); sucrose (100 mM); K-phosphate buffer, pH 7.4 (10 mM); MgCl₂ (10 mM); Na-succinate (4 mM); proline (4 mM); arginine (2 mM); serine (2 mM); and L-leucine-¹⁴C (0.5 μCi/ml, specific activity = 20 mCi/mmmole). PEP (phosphoenolpyruvate) was 5.0 mM and PK (pyruvate kinase) was 0.1 mg/ml where added. Further additions are indicated. Ammonium ion was added as the sulfate.

Additions to incubation medium	Nerve cell Glial cell mitochondria mitochondria	
	cpm/mg protein	
1.5 mM ATP	104	50
1.5 mM ATP	145	120
+ 30 mM NH ₄ ⁺	180	
1.5 mM ATP	147	90
+ 30 mM NH ₄ ⁺	229	
2.5 mM ATP, PEP, PK	97	42
3.0 mM ATP	118	
+ PEP, PK	156	115
3.0 mM ATP	132	110
1.5 mM ATP (Bicine buffer instead of Tris buffer)	83	41

dase activity is seen to approach zero at about 38% sucrose, whereas monoamine oxidase activity still remains high until about 35% sucrose, as would be expected if the region between 35–38% sucrose consists largely of synaptic nerve endings but little mitochondrial material. These findings thus demonstrate that a sucrose density gradient is capable of significant separation of the glial and neuronal mitochondria and also that the glial mitochondria can be separated from a fraction rich in synaptic nerve endings.

Density Gradient Separations of Mixed Whole Brain Mitochondria

The observations described above prompted investigations to determine whether a more gradual sucrose density gradient might be effective in separating glial and neuronal mitochondria

from a total mitochondrial fraction of whole rabbit cortex. The experiment shown in Fig. 8 shows that two well-separated peaks of cytochrome oxidase activity may be observed in such a more gradual gradient. Separation of monoamine oxidase activity into two peaks approximately congruent with the cytochrome oxidase peaks also took place. These experiments therefore indicate

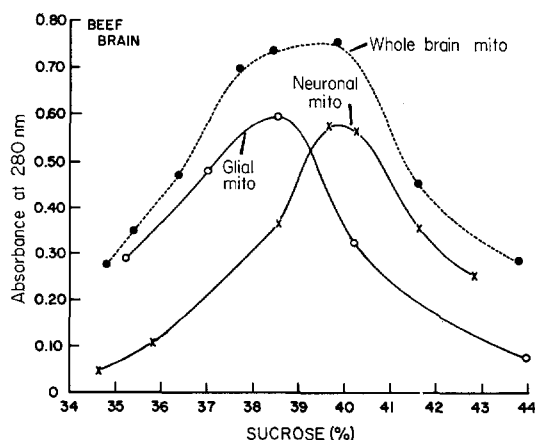


FIGURE 5 Sucrose density gradient (20–50%) profiles of mitochondria from whole brain, neuron-enriched, and glia-enriched fractions of beef cerebral cortex. The mitochondria were prepared by differential centrifugation and purified on a discontinuous sucrose gradient (0.8/1.2 M) prior to layering on the continuous gradient. Absorption at 280 nm was used as an index of protein content.

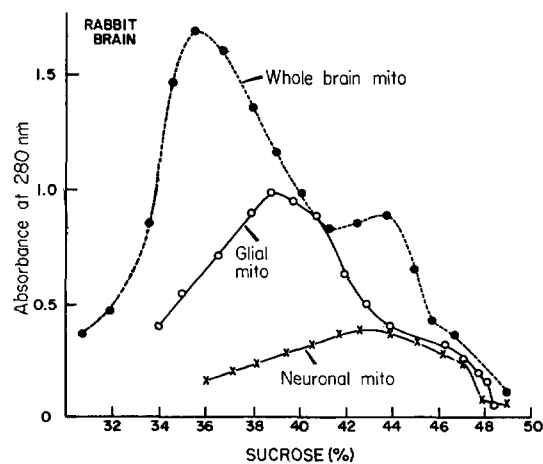


FIGURE 6 Sucrose density gradient (30–50%) profiles of mitochondria from neuron-enriched and glia-enriched fractions and of mixed mitochondria from whole rabbit cerebral cortex.

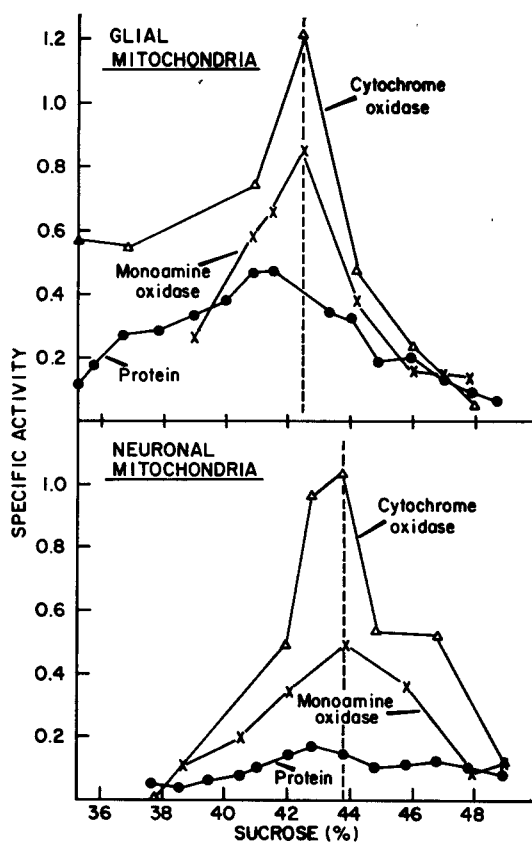


FIGURE 7 Profile of total protein and of the specific activities of cytochrome oxidase and monoamine oxidase on a sucrose density gradient (30–50%) of mitochondria isolated from neuron-enriched and glia-enriched fractions from rabbit cerebral cortex. Protein was determined by the procedure of Lowry et al. (1951).

the feasibility of refining this approach for separating neuronal from glial mitochondria. The large amount of lighter monoamine oxidase activity presumably corresponds to synaptic nerve endings. This light fraction and the heavier mitochondrial fraction were also assayed after hypotonic treatment with water. However, there was no change in the ratio of monoamine oxidase to cytochrome oxidase activity following osmotic shock.

DISCUSSION

The modifications we have introduced into the density gradient procedure (Blomstrand and Hamberger, 1969) for separation of neuronal and glial cells make possible preparation of large

amounts of neuron- and glia-enriched fractions under conditions that minimize the admixture of free cell organelles, particularly mitochondria. The gentle stirring of the minced brain tissue with a propeller has been found to be the only practical means of preparing cell dispersions in large-scale experiments involving one or more kilograms of brain tissue.

A major handicap in assessing the effectiveness of the separation methods is that only microscopic criteria can be used at present for evaluating the purity of the neuronal and glial cell fractions. Specific marker enzymes for some of the major contaminants are not available; moreover, earlier data on specific enzymatic activities observed with microtechniques on specialized areas of the brain may not be applicable to whole brain dispersions, in which neuronal and glial cells vary greatly in proportion, size, form, function, and site of origin. However, the cell bodies of the neurons were easily recognized by light microscopy as is shown in Fig. 1. The neuronal fraction was found to contain as its main contaminants some small blood capillaries and free nuclei. The morphological composition of the glial cell fraction was much more difficult to judge. However, intact astrocytes were readily identified (Fig. 2). Nuclei of both oligodendrocytes and astrocytes could also be seen. In addition, some sections also contained unidentified structures. However, the most crucial observation is that virtually no neuronal cell bodies were found in the glial fractions.

The data on enzymatic activities of glial and neuronal mitochondria, while not extensive due to limitations of material, did show a number of noteworthy points. The $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity was somewhat higher in homogenates of glial than neuronal cells; this could of course be due to a difference in the extent of contamination with synaptic nerve endings, which are rich in $\text{Na}^+ + \text{K}^+$ -stimulated ATPase activity (Kurokawa et al., 1965). In agreement with earlier investigators (Hosie, 1964, 1965; Kurokawa et al., 1965; Bradford et al., 1966) we found Na^+ and K^+ -activated ATPase to be present in brain mitochondrial fractions extensively purified by gradient centrifugation. Beattie and Basford (1968) have shown that the ATPase activity of brain mitochondria is stimulated 30–50% by 15–50 mM NaCl; this stimulation is inhibited by oligomycin but not by ouabain. In this connection, it may be noted that brain mitochondria

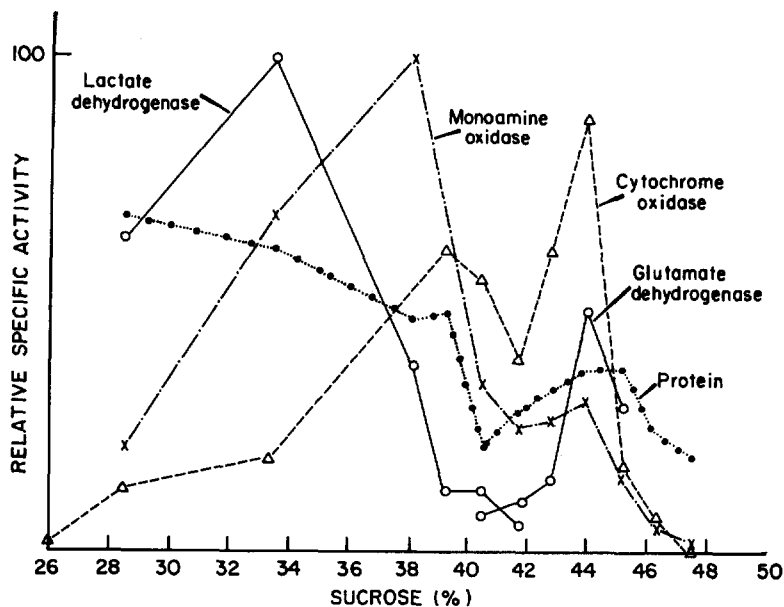


FIGURE 8 Distribution of protein and specific activities of cytochrome oxidase, monoamine oxidase, lactic dehydrogenase, and glutamate dehydrogenase on a sucrose density gradient (32–48%) of mitochondria from whole rabbit cerebral cortex. Protein was determined by the Lowry method. The dotted vertical lines indicate the points of near congruence in the peaks of cytochrome oxidase and monoamine oxidase activities at 38.7% sucrose and 44% sucrose.

contain several times more potassium and sodium than liver, kidney, and heart mitochondria (Ozawa et al., 1967). Although the role of the glial cells and their mitochondria is still obscure in this connection, a specific transport function for the mitochondrial ATPase would not be unexpected.

The respiratory rates with the different substrates tested were quite similar for neuronal and glial mitochondria. Since the amount of material required for respiratory rate studies with the oxygen electrode is relatively large, there were severe limitations to the number of measurements of respiratory rate that could be carried out. Although mitochondrial oxidation of α -glycerophosphate has been shown histochemically to be prominent in glial cells (Friede, 1966), we did not find especially high activity in the isolated glial mitochondria. Actually, Tipton and Dawson (1968) have found that α -glycerophosphate dehydrogenase activity in different regions of the brain parallels that of cytochrome oxidase.

We were unable to observe any significant difference in the monoamine oxidase:cytochrome oxidase activity ratio when comparing neuronal and glial mitochondria from beef brain. However,

mitochondria from the glia-enriched fraction of rabbit cerebral cortex were characterized by a significantly higher monoamine oxidase:cytochrome oxidase activity ratio than neuronal mitochondria (Fig. 7). In view of the fact that cytochrome oxidase is a marker for the inner mitochondrial membrane and monoamine oxidase for the outer membrane (Schnaitman et al., 1967), and the fact that the ratio of inner to outer membrane mass may be different in nerve and glial cell mitochondria, depending on the region of the brain, the large differences in the ratio of monoamine oxidase to cytochrome oxidase activity in mitochondria isolated from different portions of the brain (Weiner, 1960) may be related to differences in the relative areas of the outer and inner membranes of nerve cell and glial cell mitochondria.

The considerably higher capacity for protein synthesis shown by the mitochondria derived from the neuron-enriched fraction is one of the main points of difference between mitochondria from glial and neuronal fractions. This difference in incorporation was consistently observed despite considerable variation in incubation conditions, indicating that the observed difference is inherent

rather than the effect of some specific deficiency of cofactors in the test medium. These observations are relevant to the finding that in nerve cells the mitochondria may increase in number during increased function (Hartman, 1948; Hudson et al., 1961; Eneström and Hamberger, 1968).

The difference in apparent buoyant density of the neuronal and glial mitochondria on the continuous sucrose gradient demonstrates that these two cell types have distinct populations of mitochondria, with the neuronal mitochondria having a higher apparent buoyant density. This difference was found in the case of both beef brain and rabbit mitochondria. Isolated mitochondria that were purified on Ficoll gradients did not undergo a change in density when placed on sucrose gradients. We have also carried out experiments on recombination of neuronal, glial, and whole brain mitochondria, in which one type of mitochondria was prelabeled with a radioactive amino acid; a radioactivity peak was found in the expected position on the sucrose density gradient, thus confirming the conclusions drawn from the nonisotopic experiments.

The differences in the buoyant density of neuronal and glial mitochondria led us to investigate the sedimentation and enzyme profiles of whole brain mitochondria on sucrose density gradients, in order to determine the feasibility of separating the two types of mitochondria from a mixed population by means of density gradient centrifugations. Two discrete peaks of high cytochrome oxidase activity could be obtained in such mixtures by use of a more gradual sucrose gradient (Fig. 8). The peak of cytochrome oxidase activity observed at the lower density differs markedly from the peak of high lactic dehydrogenase activity, which was used as a marker to show the location of the synaptic nerve endings that enclose cytoplasm. A number of studies have shown that synaptic nerve ending fractions are not homogeneous (De Robertis et al., 1963; Fonnum, 1968; Whittaker, 1968). However, since the lactic dehydrogenase activity is low at the higher cytochrome oxidase peak, it is probable that the latter is due to free glial mitochondria. The buoyant density of the heavier peak of cytochrome oxidase activity corresponds to that found for mitochondria isolated from the neuronal fraction.

As is seen in Fig. 8 the ratio monoamine oxidase: cytochrome oxidase increased significantly as

density decreased. This difference was also found on examination of a mitochondrial and a synaptosomal fraction, obtained according to Gray and Whittaker (1962). Even strong hypoosmotic shock, known to burst the nerve-ending membranes (De Robertis et al., 1963) and to release the entrapped mitochondria, failed to alter the ratio of monoamine oxidase to cytochrome oxidase. It therefore seems improbable that the observed distribution of monoamine oxidase and cytochrome oxidase is the result of removal of the outer mitochondrial membrane and its appearance as an artifact in the synaptosomal fraction.

Several observations indicate that cytochrome oxidase activity parallels the activity of succinic dehydrogenase in a number of tissues including brain (Pette et al., 1962; Shimizu and Morikawa, 1957). The ratio of monoamine oxidase to either of these enzymes is known to vary considerably from one brain region to another (Weiner, 1960; Tipton and Dawson, 1968). These variations may be caused by regional differences in the neuronal and/or glial populations, or differences between neuronal perikarya, nerve cell processes, and neuroglial cells in a given region (Weiner, 1960). The recent observations by Hajos and Kerpel-Fronius (1969) indicate a marked difference in enzyme activity between perikaryal and nerve-ending mitochondria. Our results do not entirely agree with earlier data showing a close parallelism of succinic dehydrogenase activity with monoamine oxidase activity when brain mitochondria are subfractionated on a continuous sucrose gradient (Rodriguez De Lores Arnaiz and De Robertis, 1962; Neidle et al., 1969). Rather, our findings suggest that the variations in the cytochrome oxidase (or succinic dehydrogenase): monoamine oxidase activity ratios are due to differences in the structure of mitochondria in nerve cell bodies, nerve endings, and glial cells, particularly the ratio of inner to outer membrane mass.

Although our results to date give only a fragmentary view of the enzymatic and metabolic characteristics of the mitochondria from neurons and glial cells, further work can be expected to yield more penetrating information relevant to the functional and metabolic interplay between these two cell types.

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